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In vivo enzymology: ^{13}C NMR measurement of a kinetic isotope effect for methanol oxidation in *Methylosinus trichosporium* OB3b

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The competitive oxidation of $^{13}CH_3OH$ and $^{13}CD_3OH$ has been observed using in vivo ^{13}C NMR spectroscopy. Simultaneous ^{1}H and ^{2}H decoupling gave isotopically shifted ^{13}C singlets for the two methanol isotopomers. The measured enzymic isotope effect, k_H/k_D is approx. 1.8, indicating that C–H bond cleavage is not rate-determining.

Enzyme kinetics; NMR; Methanol oxidation; Isotope effect; Formaldehyde production

1. INTRODUCTION

Kinetic isotope effects can provide important insights into the details of enzyme reaction mechanisms [1,2]. Usually such effects are measured for isolated enzymes in vitro, but it should be instructive to measure them in vivo, under more physiological conditions. Isotope effects can be determined by either competitive or non-competitive methods [2]. In the latter, which are the most common, the experiment is carried out separately for the labelled and unlabelled substrates under 'identical' conditions. There is no need for the assay technique to distinguish labelled from unlabelled substrate. In practice, however, identity of experimental conditions is a demanding prerequisite which is very difficult to achieve; the isotope effects of interest are often smaller than experimental error. This will be particularly true for living systems or for secondary isotope effects. By contrast, a competitive experiment only requires accurate relative rates of reaction, e.g. k_H/k_D, in a mixture of two isotopomers of the substrate, but it must be able to discriminate between the two forms of the substrate or product.

We show here that deuterium-induced shifts [3] in ^{13}C NMR spectra can be exploited to allow simultaneous detection, and hence measurement of the relative concentrations, of $^{13}CH_3OH$ and $^{13}CD_3OH$ in a mixture. We illustrate the application of this technique in the measurement of the isotope effect for methanol oxidation in the obligate methanotroph *Methylosinus trichosporium* OB3b. In vivo ^{13}C observations of the oxidation pathway from methanol to carbon dioxide via formaldehyde and formate in this and related organisms have been reported previously [4,5].

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2. EXPERIMENTAL

$^{13}CH_3OH$ (90%, ^{13}C) was obtained from Aldrich, and $^{13}CD_3OH$ (90% ^{13}C, 98% D) from Amersham International. The organism *M. trichosporium* OB3b was grown on methane, under oxygen limitation in continuous culture (D = 0.04 h^{-1}) in a minimal medium containing 10 μM

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$CuSO_4$; it was prepared for NMR spectroscopy as described in [4]. An equimolar mixture of $^{13}CH_3OH$ and $^{13}CD_3OH$ was added to give a total concentration of 17 mM methanol. The cell suspension, in a 10 mm diameter tube fitted with aeration tube [4], was placed in the spectrometer and thermostatted to 30°C. Spectrum acquisition was started, and metabolism was initiated by bubbling air into the suspension.

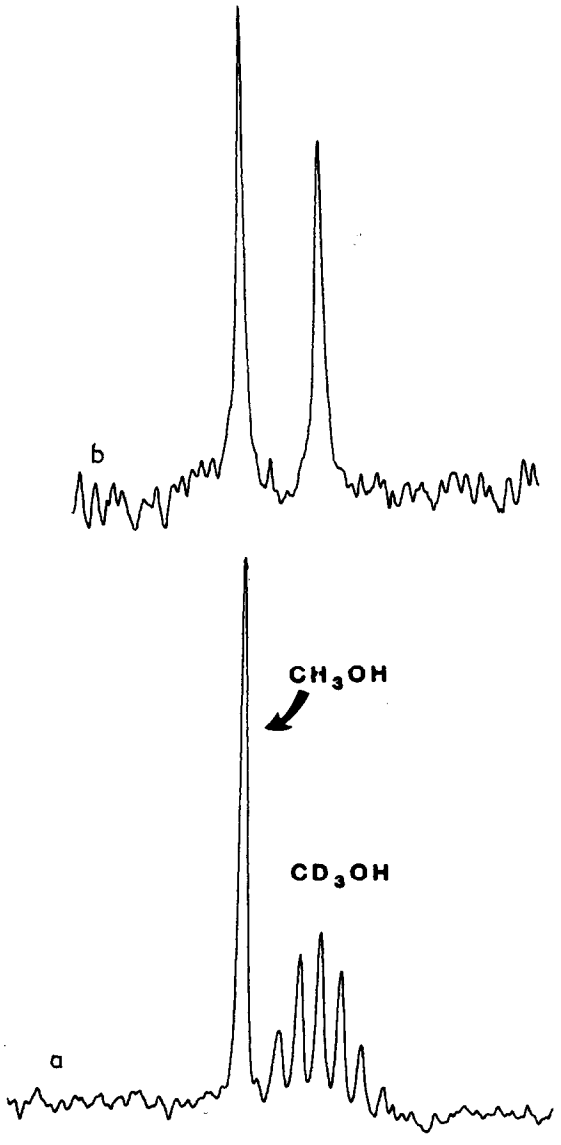

Fig.1. 100.6 MHz ^{13}C spectra of an aqueous mixture of $^{13}CH_3OH$ and $^{13}CD_3OH$: (a) ^1H decoupled; (b) ^1H and ^2H decoupled.

100.6 MHz ^{13}C spectra were acquired unlocked on a Bruker WH400 instrument using WALTZ-16 ^1H decoupling: field strength 2 kHz, power <1 W [6]. Simultaneously, gated deuterium decoupling was achieved through the lock coil using swept square wave modulation and 5 W power; the deuterium irradiation was centred on the methanol frequency to maximise decoupling efficiency.

Successive spectra were obtained by co-adding 40 transients with 4 s recycling time. 10 Hz exponential line broadening was applied to improve the signal-to-noise ratio.

3. RESULTS

Fig.1 shows the ^{13}C spectrum of a mixture of $^{13}CH_3OH$ and $^{13}CD_3OH$ acquired with proton decoupling only and with simultaneous proton and deuterium coupling. The chemical shift of $^{13}CH_3OH$ is 50.2 ppm, while CD_3OH resonates 0.8 ppm to high field, at 49.4 ppm. In the absence of deuterium decoupling the $^{13}CD_3OH$ resonance is a septet (J_{CD} = 21 Hz); deuterium decoupling improves the sensitivity of detection substantially, allowing finer time resolution in kinetic experiments. Time course spectra were obtained with a resolution of 160 s.

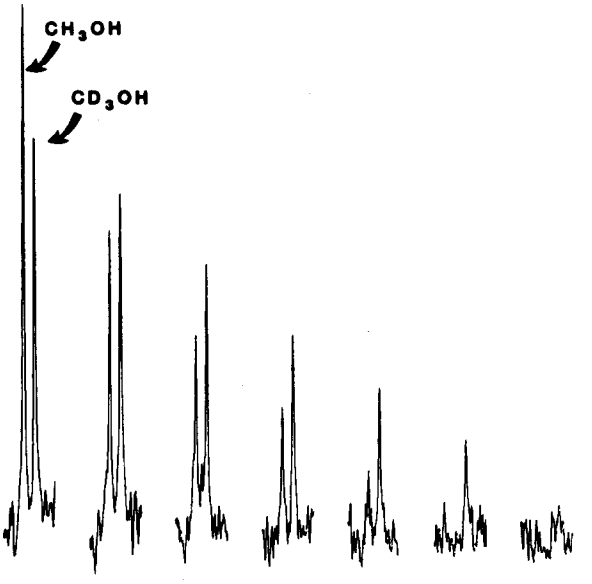

Fig.2. Time course of methanol oxidation by an aerated suspension of *Methylosinus trichosporium* OB3b. Spectra were acquired for 160 s each; alternate spectra are shown.

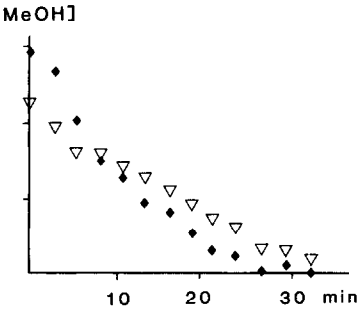

Fig.3. Time course of methanol oxidation. (♦) CH_3OH, (▽) CD_3OH.

Methanol oxidation was initiated by aeration of the bacterial suspension in the spectrometer, a representative set of spectra being shown in fig.2. It is clear that the deuterated isotopomer is oxidised more slowly. The half-life for consumption of $^{13}CH_3OH$ was approx. 9 min (fig.3), while for $^{13}CD_3OH$ it was approx. 17 min, giving a value of ~1.8 for k_H/k_D. (The decrease in methanol consumption rate with time, which may be a result of inhibition by product formaldehyde, coupled with the poor signal-to-noise ratio, precludes more detailed kinetic analysis in this particular system.)

4. DISCUSSION

The use of NMR spectroscopy to measure the kinetics of enzymic reactions in whole cells or tissues is well established; indeed, saturation transfer techniques even allow the measurement of enzyme turnover rates at equilibrium [7,8]. However, we are aware of no previous reports of enzymic kinetic isotope effects that have been determined by NMR; this may be because the relatively low absolute precision of such experiments has so far prevented non-competitive experiments being successfully carried out. The competitive approach described here, which has been used to observe α- vs β-anomeric preferences in whole cells [9], will allow measurement of relatively small secondary isotope effects. Since we are only interested in relative concentrations of the two species, factors such as differential NOEs or linewidths and partial saturation are not relevant, providing that they remain constant throughout the experiment.

Other approaches to in vivo kinetic isotope effects usually rely on non-kinetic techniques such as site-selective enrichment [10–12] or 'metabolic switching' [13]. This switching occurs when the existence of a large isotope effect on one branch of a multiple-choice pathway leads to a different product distribution for labelled and unlabelled substrates.

The relatively small value obtained for the kinetic isotope effect for methanol oxidation by *M. trichosporium* implies that C-H bond cleavage is not the rate-determining step in overall enzyme turnover; if it were, a value of 6–8 would have been expected [2]. These results, then, are but a first step in elucidating the details of the oxidation process.

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REFERENCES

[1] Jencks, W.P. (1969) Catalysis in Chemistry and Enzymology, McGraw Hill, pp.243–281.
[2] Northrop, D.B. (1982) Methods Enzymol. 87, 607-625.
[3] Walker, T.E. and Wageman, W.E. (1986) Magn. Reson. Chem. 24, 157–161.
[4] Cornish, A., Nicholls, K.M., Hunter, B.K., Aston, W.J., Higgins, I.J. and Sanders, J.K.M. (1984) J. Gen. Microbiol. 130, 2565–2575.
[5] Santos, H. and Turner, D.L. (1986) J. Magn. Reson. 68, 345–349.
[6] Shaka, A.J., Keeler, J.H., Frenkiel, T. and Freeman, R. (1983) J. Magn. Reson. 52, 335–338.
[7] Alger, J.R. and Shulman, R.G. (1984) Q. Rev. Biophys. 17, 83–124.
[8] Boyd, J., Brindle, K.M., Campbell, I.D. and Radda, G.K. (1984) J. Magn. Reson. 60, 149–155.
[9] Barrow, K.D., Rogers, P.L. and Smith, G.M. (1986) Eur. J. Biochem. 157, 195–202.
[10] Damgaard, S.E. (1981) Biochemistry 20, 5662–5669.
[11] Lu, A.Y.H., Harada, N. and Miwa, G.T. (1984) Xenobiotica 14, 19–26.
[12] Martin, G.J., Zhang, B.L., Naulet, N. and Martin, M.L. (1986) J. Am. Chem. Soc. 108, 5116–5122.
[13] Foster, A.B. (1984) Trends Pharmacol. Sci. 5, 524–527.